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ORIGINAL ARTICLE

Identification of a novel cyanobacterial group as active diazotrophs in a coastal microbial mat using NanoSIMS analysis

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N_2 fixation is a key process in photosynthetic microbial mats to support the nitrogen demands associated with primary production. Despite its importance, groups that actively fix N_2 and contribute to the input of organic N in these ecosystems still remain largely unclear. To investigate the active diazotrophic community in microbial mats from the Elkhorn Slough estuary, Monterey Bay, CA, USA, we conducted an extensive combined approach, including biogeochemical, molecular and high-resolution secondary ion mass spectrometry (NanoSIMS) analyses. Detailed analysis of dinitrogenase reductase (*nifH*) transcript clone libraries from mat samples that fixed N_2 at night indicated that cyanobacterial *nifH* transcripts were abundant and formed a novel monophyletic lineage. Independent NanoSIMS analysis of $^{15}N_2$-incubated samples revealed significant incorporation of ^{15}N into small, non-heterocystous cyanobacterial filaments. Mat-derived enrichment cultures yielded a unicyanobacterial culture with similar filaments (named Elkhorn Slough Filamentous Cyanobacterium-1 (ESFC-1)) that contained *nifH* gene sequences grouping with the novel cyanobacterial lineage identified in the transcript clone libraries, displaying up to 100% amino-acid sequence identity. The 16S rRNA gene sequence recovered from this enrichment allowed for the identification of related sequences from Elkhorn Slough mats and revealed great sequence diversity in this cluster. Furthermore, by combining $^{15}N_2$ tracer experiments, fluorescence *in situ* hybridization and NanoSIMS, *in situ* N_2 fixation activity by the novel ESFC-1 group was demonstrated, suggesting that this group may be the most active cyanobacterial diazotroph in the Elkhorn Slough mat. Pyrotag sequences affiliated with ESFC-1 were recovered from mat samples throughout 2009, demonstrating the prevalence of this group. This work illustrates that combining standard and single-cell analyses can link phylogeny and function to identify previously unknown key functional groups in complex ecosystems.

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Introduction

Photosynthetic microbial mats are ecosystems with high densities of functional and phylogenetic diversity, resulting in a strongly coupled cycling of elements (Canfield and Des Marais, 1993; Bebout *et al.*, 1994; Ley *et al.*, 2006). Therefore, these ecosystems have been studied extensively to gain fundamental insights into global processes with implications ranging from the early evolution of microorganisms and metabolic diversity to microbial interactions and nutrient cycling (Des Marais, 1990; Paerl *et al.*, 2000; Des Marais, 2003). The cycling and flux of carbon, sulfur and nitrogen are particularly intertwined in these mats. The availability of nitrogen determines overall mat productivity, because photosynthetic primary production creates a high demand for fixed nitrogen, which requires high rates of N_2 fixation (Herbert, 1999). However, the

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distribution of N_2 fixation activity among phylogenetically and functionally diverse microorganisms in the mats remains largely unclear.

In most microbial mats, the uppermost layer is visually dominated by unicellular and non-heterocystous filamentous cyanobacteria, such as *Microcoleus* or *Lyngbya* spp. (D'Amelio *et al.*, 1989). N_2 fixation in these mats has a distinct diel pattern: N_2 fixation is largely absent during the day, increases after sunset and is maximal at night or in the early morning prior to sunrise (Stal *et al.*, 1984; Bebout *et al.*, 1987, 1994; Omoregie *et al.*, 2004a, b). Daytime suppression of N_2 fixation has been attributed to high levels of O_2 from oxygenic photosynthesis, which leads to inhibition of the O_2-sensitive nitrogenase (Bothe, 1982). The mats in the Elkhorn Slough estuary (Moss Landing, CA, USA), that were the focus of this study are dominated by filamentous cyanobacteria, in particular by *Microcoleus* spp. (Burow *et al.*, 2011). However, the N_2 fixation activity pattern and the diazotrophic community in Elkhorn Slough mats were so far unknown.

Traditionally, cyanobacteria were thought to have a major role in the total N_2 fixation in microbial mats (Paerl *et al.*, 1991; Bebout *et al.*, 1993). A few cyanobacteria that were isolated from microbial mats fixed N_2 in cultivation-based studies and corroborated this hypothesis (Stal and Krumbein, 1981; Paerl *et al.*, 1991). However, concern of general cultivation bias has motivated researchers to pursue cultivation-independent methods to gain more comprehensive insight of the diazotrophic community in complex ecosystems (Zehr *et al.*, 1995).

N_2 fixation is mediated by the nitrogenase enzyme complex, consisting of *nifH*-encoded dinitrogenase reductase, which transfers electrons to dinitrogenase, encoded by *nifD* and *nifK*, ultimately catalyzing the reduction of N_2 to NH_3. This unique process has been studied at different levels using various methods such as acetylene reduction assay (ARA) and *nifH* surveys. The fortuitous transformation of acetylene to ethylene by nitrogenase makes the ARA a useful, indirect measure for nitrogenase activity in cultures as well as in complex communities (Stewart *et al.*, 1967). The high sensitivity of the assay has enabled measurements of rapid changes in N_2 fixation activity in response to rapidly changing environmental conditions. Employment of this technique in diel cycle studies of microbial mats has provided invaluable insights into the patterns of N_2 fixation depending on irradiance and thus O_2 concentrations, such as the above-mentioned patterns for *Microcoleus*- and *Lyngbya* spp.-dominated mats (Stal *et al.*, 1984; Bebout *et al.*, 1987; Bebout *et al.*, 1994; Paerl *et al.*, 1996). More recently, the *nifH* gene has been used as a phylogenetic and functional marker for N_2 fixation and allows investigating the phylogenetic distribution of the genetic potential for N_2 fixation in complex microbial communities. Surveys of *nifH* in microbial mats suggested that heterotrophic bacteria might also have an important role in microbial mat N_2 fixation in addition to cyanobacteria (Zehr *et al.*, 1995; Steppe *et al.*, 1996; Zehr *et al.*, 2003; Omoregie *et al.*, 2004a; Severin *et al.*, 2010; Severin and Stal, 2010b). Analysis and quantification of *nifH* transcripts have helped to identify the fraction of diazotrophs actively expressing this essential gene for N_2 fixation and has given insights into gene-expression dynamics in the environment (Omoregie *et al.*, 2004b; Moisander *et al.*, 2006; Severin and Stal, 2010a). Previous studies (Steunou *et al.*, 2008; Severin and Stal, 2010a) have revealed discrepancies between the expression of *nifH* by diazotrophic groups and nitrogenase activity patterns measured by acetylene reduction, illustrating that gene expression does not necessarily correspond to activity.

In contrast to the above-mentioned methods, stable isotope probing with $^{15}N_2$ provides a direct and unambigious measure of N_2 incorporation activity (Montoya *et al.*, 1996). While ^{15}N incorporation is measured in bulk by isotope ratio mass spectrometry (IRMS), secondary ion mass spectrometry (SIMS) and the recently developed CAMECA NanoSIMS for high-resolution SIMS have enabled the connection of ecosystem level processes to activities at the level of single cells. These technologies have been used for the stable isotope probing of the metabolic activities of cell aggregates (Orphan *et al.*, 2001) or single cells (Lechene *et al.*, 2006; Popa *et al.*, 2007; Finzi-Hart *et al.*, 2009; Ploug *et al.*, 2010; Foster *et al.*, 2011), respectively. In combination with fluorescence *in situ* hybridization (FISH) targeting 16S rRNA, SIMS studies enable direct linkages of phylogeny to function in natural communities (Orphan *et al.*, 2001; Behrens *et al.*, 2008; Li *et al.*, 2008; Musat *et al.*, 2008; Halm *et al.*, 2009).

In this study, we used (to the best of our knowledge) an unprecedented breadth of methods—nitrogenase activity measurements, analysis of *nifH* gene diversity and expression, $^{15}N_2$ tracer experiments, NanoSIMS, catalyzed reporter deposition (CARD)-FISH and cultivation experiments—to identify active N_2-fixing microorganisms in a complex microbial mat ecosystem. By this combined approach, we were able to characterize a novel group of diazotrophic cyanobacteria in Elkhorn Slough microbial mats, and demonstrated their ecophysiological importance in N_2 fixation.

Materials and methods

Study site

The sampling site is located in the Elkhorn Slough estuary at 36°48′46.61″N and 121°47′4.89″W. The Elkhorn Slough is a shallow seasonal estuary that extends inland 11 km from Monterey Bay with mixed semidiurnal tides; tidal exchange and sporadic surface water input during winter rainy seasons

are the main water transport mechanisms (Chapin and Johnsin, 2004).

Mat sampling and diel cycle studies setup
Microbial mats collected at Elkhorn Slough (10 pieces of ca. 144 cm^2 of 2 cm thickness including a 1 cm sediment layer) were sampled on 20 October 2009 and transported to a greenhouse facility transparent to ultraviolet radiation at NASA Ames Research Center within 1–2 h. In the greenhouse, mat pieces were placed in acrylic aquaria transparent to ultraviolet radiation and covered with *in situ* water (circulated and aerated) for ca. 20 h before the beginning of a diel cycle study (starting at 1200 hours and ending at 1500 hours the following day). Two successive diel cycle studies with the same mats were carried out (21/22 and 23/24 October 2009) under natural solar irradiance, and the water temperature was kept constant at ca. 18 °C (*in situ* average).

Biogeochemical analysis (ARAs and $^{15}N_2$ incubations)
Nitrogenase activity was measured with the ARA as previously described (Bebout *et al.*, 1993). Mat cores (10 mm diameter, 10 mm thick) were sampled in triplicate every 3 h, and subsequently incubated with acetylene for 3 h. Triplicate water samples without mat served as negative controls. Ethylene was quantified in a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan). For measuring the depth distribution of nitrogenase activity in the mats, triplicate mat cores of 10 mm diameter and 10 mm thickness were horizontally sectioned into three layers (uppermost layer 0–2 mm depth, second layer 3–6 mm and third layer of 7–10 mm depth) and the layers were separately incubated as mentioned above.

To measure $^{15}N_2$ incorporation, mat cores of 10 mm diameter and 10 mm thickness were transferred to a 14 ml serum vial, covered with 1 ml of *in situ* water, capped with gas-tight rubber stoppers and 8 ml of the headspace was exchanged with $^{15}N_2$ gas (98+ atom% $^{15}N_2$; Cambridge Isotope Laboratories, Andover, MA, USA). Mats were incubated for 10 h in the dark (2030 hours until 0630 hours the next day), and subsequently, half of the mat cores were sectioned for bulk isotope analysis in the same depth intervals as mentioned above. The other portions of the sectioned cores were preserved for NanoSIMS analysis by fixation in 4% paraformaldehyde for ca. 16 h at 4 °C. Fixed cores were washed twice in 1× phosphate-buffered saline (PBS) (pH 7.6) and stored in PBS/ethanol (40/60, vol/vol) at −20 °C for further analysis. Unlabeled mat sections served as controls. Isotope ratios for $^{15}N/^{14}N$ were determined by IRMS (ANCA-IRMS; PDZ Europa Limited, Crewe, England) at the University of California, Berkeley. Additionally, mat cores were first sectioned and then incubated with $^{15}N_2$ to verify the depth distribution of N_2 fixation by IRMS.

IRMS and ARA data of vertical sections were analyzed for statistical differences using an analysis of variance with a Tukey's HSD mean separation at $P<0.05$ using the R program version 2.13.1 (http://www.r-project.org/index.html).

Companion samples for nucleic acid extraction and subsequent molecular analysis (six independent cores of 10 mm diamater and 10 mm thickness per time point) were flash frozen in liquid nitrogen and stored at −80 °C.

Cultivation and $^{15}N_2$ incubation experiments of Elkhorn Slough Filamentous Cyanobacterium-1 (ESFC-1)
Cyanobacteria were enriched from microbial mat samples as described previously (Prufert-Bebout and Garcia-Pichel, 1994). Media formulations and enrichment conditions are described in the Supplementary Information. After enrichment, the cyanobacterial cultures were assessed for N_2 fixation activity after three washes in N-free media (ASN-) and pre-incubated in ASN- for 4 days. Cultures were then transferred into 14 ml serum vials, which were filled with fresh ASN- media to eliminate gas headspace. The vials were capped with gas-tight rubber stoppers and 35 µl of $^{15}N_2$ was added, whereas an equal volume of medium was vented with a needle. Vials were incubated at 22 °C for 24 h (dark/light cycle, 8 h/16 h, light intensity ca. 40 µmol photons m^{-2} s^{-1}). At the end of the incubation, the cyanobacterial biomass was rinsed in 1× PBS and frozen at −80 °C for IRMS analysis.

Molecular analysis
Detailed information about DNA and RNA extractions can be found in Supplementary Information. Briefly, RNA and DNA of microbial mats were co-extracted from the uppermost 2 mm of three pooled mat cores by combining phenol–chloroform extraction with parts of the RNeasyMini and QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), respectively. RNA was reverse transcribed into single-stranded cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

Detailed information about the construction of 16S rRNA gene/transcripts and *nifH* gene/transcript clone libraries can be found in the Supplementary Information. In summary, (1) a 16S rRNA gene clone library was constructed from the cyanobacterial enrichment culture ESFC-1 (total of 36 sequences). (2) general 16S rRNA clone libraries of the upper 2 mm of Elkhorn Slough mats were generated from mat samples collected on 12/13 January 2009. The following clone libraries were constructed: 12 January, 2100 (total of 329 sequences) and 13 January, 0700 hours (total of 243 sequences). (3) a *nifH* gene clone library was generated from the cyanobacterial enrichment culture ESFC-1 (total of

39 sequences) (4) clone libraries of the *nifH* genes (DNA) and transcripts (cDNA) were constructed from the uppermost 2 mm of mat cores sampled during two consecutive diels: 21 October 2009, at 2250 hours (DNA: 93 sequences and cDNA: 92 sequences) and 24 October 2009, at 0310 hours (DNA: 75 sequences and cDNA: 88 sequences). Sequence analyses of the clone libraries listed above are described in Supplementary Information.

Amplicons of the 16S rRNA V8 hypervariable region were constructed from seven time points in the year 2009 (13 January, 30 April, 1 July, 19 August, 16 September, 21 October and 13 November 2009) (see Supplementary Information for more detailed description). Analysis of the V8 amplicon sequences is described in Supplementary Information.

Sample preparation for NanoSIMS analysis

Material from the uppermost 2 mm of fixed microbial mats from the October 2009 samples ($^{15}N_2$-incubated and sampled for natural abundances) were transferred with tweezers onto silicon wafers (Ted Pella, Redding, CA, USA), teased apart and attached by drying. In experiments where CARD-FISH was combined with NanoSIMS analysis, wafers were coated with VectaBond (Vector Laboratories, Burlingame, CA, USA). Wafers were mapped with reflected light and scanning electron microscopy (SEM) for orientation in the NanoSIMS. SEM images of higher magnification were also collected to match increased $^{15}N/^{14}N$ ratios subsequently measured by NanoSIMS with microbial cells. Higher magnification images were also taken from filamentous cyanobacteria to ensure that the investigated regions of cyanobacterial filaments were free of attached microorganisms and that increased $^{15}N/^{14}N$ ratios could be attributed to the cyanobacterial bacterial filament and not to associated epibionts.

CARD-FISH for NanoSIMS analysis of ESFC-1 filaments in mat samples

Design and optimization of ESFC-1-specific oligonucleotide probes are described in Supplementary Information. CARD-FISH was conducted as described previously (Pernthaler *et al.*, 2002) with hybridizations conducted at 46 °C and washing at 48 °C. Hybridization was conducted on silicon wafers coated with VectaBond; embedding in agarose was omitted. Hybridizations were performed with the following probes specific for the ESFC-1 cluster: ESFC1_172 and ESFC1_177 (Supplementary Table 1), and with NON338 (Wallner *et al.*, 1993) as a negative control and EUBI-III as positive control probe (Amann *et al.*, 1990; Daims *et al.*, 1999). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Wafers were mapped with epifluorescence microscopy, ESFC-1 filaments were identified and their location on the wafer imaged. Wafers were mapped for NanoSIMS analysis as above-mentioned.

NanoSIMS

SIMS was performed at Lawrence Livermore National Laboratory using a Cameca NanoSIMS 50 (Gennevilliers Cedex, France) as previously described (Popa *et al.*, 2007). Secondary ions $^{12}C^{14}N$ and $^{12}C^{15}N$ were detected by pulse counting to generate 10–20 serial quantitative secondary ion images (that is, layers). Samples were also imaged simultaneously by secondary electrons. Samples were sputtered to a depth of ~100 nm to achieve sputtering equilibrium before collecting data (Ghosal *et al.*, 2008). The depth of analysis during a measurement was between 50 and 200 nm. Measurements were repeated on selected cells to ensure measurement accuracy. Selected samples were also sputtered at high-beam currents (~1 nA) between repeat measurements to determine if isotopic composition changed; no significant changes were found with cell depth. Natural abundance samples of the filamentous cyanobacteria were run and analyzed, and those values were used to correct the values of $^{15}N_2$-incubated samples.

Data were processed as quantitative isotopic ratio images using custom software (LIMAGE, L Nittler, Carnegie Institution of Washington), and were corrected for detector dead-time and image shift from layer to layer. Regions of interest (ROIs) were defined in each image, and the isotopic ratio for each ROI were calculated by averaging over all of the replicate layers. For filamentous cyanobacteria, typically 3–10 connected cells within a filament were analyzed, and the isotopic ratio displayed in the Results section for the filamentous cyanobacteria is the average ratio of all filaments with standard error. Cells were analyzed for significant enrichment using a one-tailed Student *t*-test with equal variance.

Data are presented as $^{15}N/^{14}N$ ratio values and $\delta^{15}N$ values (calculated after equation (1)) and are presented as means ± standard error (SE). All reported enrichment values are corrected with natural abundance values.

$$\delta^{15}\mathrm{N} = (R_\mathrm{f}/0.00367 - 1)\times 1000 \qquad (1)$$

For a preliminary estimation of the contribution of small filamentous cyanobacteria and single cells to the observed ^{15}N incorporation, we analyzed 15 small filamentous cyanobacteria and 42 single cells in a total of 30 images of each 900–2500 μm^2 size. The $^{15}N/^{14}N$ ratio of enriched cells and filaments was measured and the atom percent enrichment (APE) of the ROI was calculated according to equation (2).

$$\mathrm{APE} = [R_\mathrm{f}/(R_\mathrm{f}+1) - R_\mathrm{i}/(R_\mathrm{i}+1)]\times 100\% \qquad (2)$$

The cell area of enriched single cells and filaments were measured based on the secondary electron

image, and the ^{15}N enrichment was calculated as APE per cell area or filament area. The origin of enriched signals was confirmed with SEM images that were collected prior to NanoSIMS analysis.

Nucleotide accession numbers

16S rRNA gene and transcript sequences obtained in this study are deposited under GenBank accession numbers JQ013010–JQ013029. Sequences of *nifH* genes and transcripts are deposited under GenBank accession numbers JQ013030–JQ013038.

Results

N_2 fixation patterns in Elkhorn Slough cyanobacterial mats

Cyanobacterial mat samples were collected near the mouth of the Elkhorn Slough on 20 October 2009. Microscopic examination of the mats revealed that the upper green layer was dominated by filamentous, non-heterocystous cyanobacteria, primarily by *Microcoleus* spp.; *Oscillatoria* spp., smaller filamentous cyanobacteria and *Lyngbya* spp. were also observed (Supplementary Figure 1).

Nitrogen fixation has not been studied previously in Elkhorn Slough mats. To elucidate the N_2 fixation patterns in these mats, mat samples were assayed for nitrogenase activity and incorporation of fixed N_2 using acetylene and $^{15}N_2$, respectively, as substrates (Bebout *et al.*, 1993; Montoya *et al.*, 1996). ARAs with complete mat cores in two successive diel cycles revealed that nitrogenase activity was more than 8-fold higher at night relative to the daytime (Figure 1). The maximum daytime nitrogenase activity was 3.7 μmol $C_2H_4\,m^{-2}\,h^{-1}$ on 21/22 October and 4.2 μmol $C_2H_4\,m^{-2}\,h^{-1}$ on 23/24 October. The nighttime activity reached 30.2 μmol $C_2H_4\,m^{-2}\,h^{-1}$ and 62.4 μmol $C_2H_4\,m^{-2}\,h^{-1}$ on 21/22 and 23/24 October, respectively. These values are within the activity range of previously studied mats from Bird Shoal, North Carolina, and Guerrero Negro, Mexico (9 μmol $C_2H_4\,m^{-2}\,h^{-1}$ to >600 μmol $C_2H_4\,m^{-2}\,h^{-1}$), which are dominated by similar cyanobacteria (Bebout *et al.*, 1993, 1994; Omoregie *et al.*, 2004a).

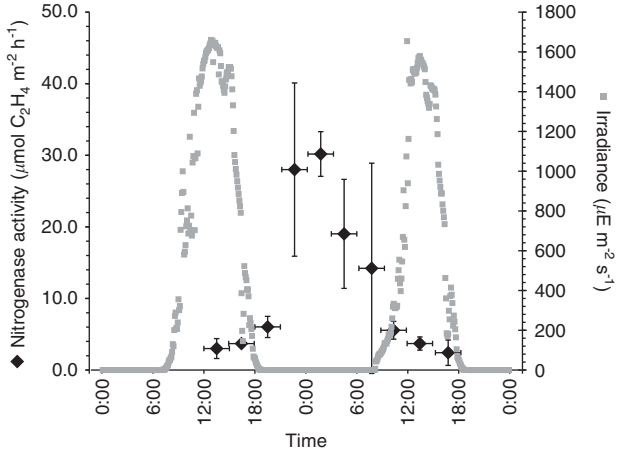

Figure 1 Nitrogenase activity measured in a diel cycle on 21/22 October 2009 of Elkhorn Slough microbial mats. ARAs were conducted with entire mat cores of 10 mm diameter and 10 mm thickness.

Further ARAs on layer-separated mat samples obtained in 2009 showed that >95% of the potential nitrogenase activity measured in the whole mat was recovered in the upper 2 mm of the ca. 1-cm-thick mats (Supplementary Figure 2). The uppermost layer (0–2 mm) had significantly higher activities than the other two deeper layers ($P<0.013$, 0–2 mm versus 3–6 mm and $P<0.013$, 0–2 mm versus 7–10 mm) (Supplementary Figure 3). In contrast, the comparison of 3–6 mm versus 7–10 mm did not show significant differences in activity ($P<0.999$). When incubated with $^{15}N_2$ in the dark, IRMS measurements performed on these layer-seperated mat samples confirmed that the uppermost layer was significantly enriched in ^{15}N relative to the 3–6 mm ($P<0.001$) and 7–10 mm ($P<0.001$) sections (Supplementary Figure 4), whereas no difference was observed comparing the 3–6 mm and 7–10 mm layers ($P<0.998$). After a 10–11 h incubation, the $^{15}N/^{14}N$ ratio in the upper layer (0–2 mm) reached $0.00424 \pm 9.2 \times 10^{-5}$ (156.2 ± 25.2‰), compared with $0.00368 \pm 2.5 \times 10^{-6}$ (3.0 ± 0.7‰) in the 3–6 mm sections and $0.00368 \pm 3.5 \times 10^{-6}$ (4.1 ± 1.0‰) in the 7–10 mm sections (Supplementary Figure 2). Therefore, the investigation of diazotrophs in Elkhorn Slough mats focused on these uppermost 2 mm.

NanoSIMS analysis of Elkhorn Slough mat microbial community

The upper layers of the ^{15}N-labeled October 2009 nighttime samples were also analyzed by NanoSIMS in order to assess ^{15}N enrichment by different cell morphotypes. Significant and high ^{15}N enrichments were observed in small cyanobacterial filaments ($P<0.001$), which were ⩽150 μm in length and composed of individual cells that were ca. 3 μm long and ca. 2 μm wide. Enrichment was also observed in multiple single cells ($P<0.001$). The enriched small filaments had a $^{15}N/^{14}N$ ratio of 0.01436 ± 0.00176 (2912.9 ± 480.8‰, $n=15$), and enriched single cells had a $^{15}N/^{14}N$ ratio of 0.03519 ± 0.00807 (8588.6 ± 2200.1‰, $n=42$) (Figure 2). In contrast, filaments of the abundant cyanobacterial group *Microcoleus* spp. and of *Oscillatoria* spp. showed very low levels of enrichment ($^{15}N/^{14}N = 0.00382 \pm 2.4 \times 10^{-5}$ or 40.9 ± 6.5‰, $n=26$ ($P<0.001$) and $^{15}N/^{14}N$ $0.00373 \pm 1.9 \times 10^{-5}$ or 17.1 ± 5.1‰, $n=10$ ($P<0.005$), respectively). For a preliminary estimation of the contribution of the small filamentous cyanobacteria and single cells to the observed ^{15}N incorporation in the upper mat layer, cellular ^{15}N enrichments were analyzed in over 30 images of 900–2500 μm² size each. Based on this screen, >80% of the total incorporated ^{15}N was

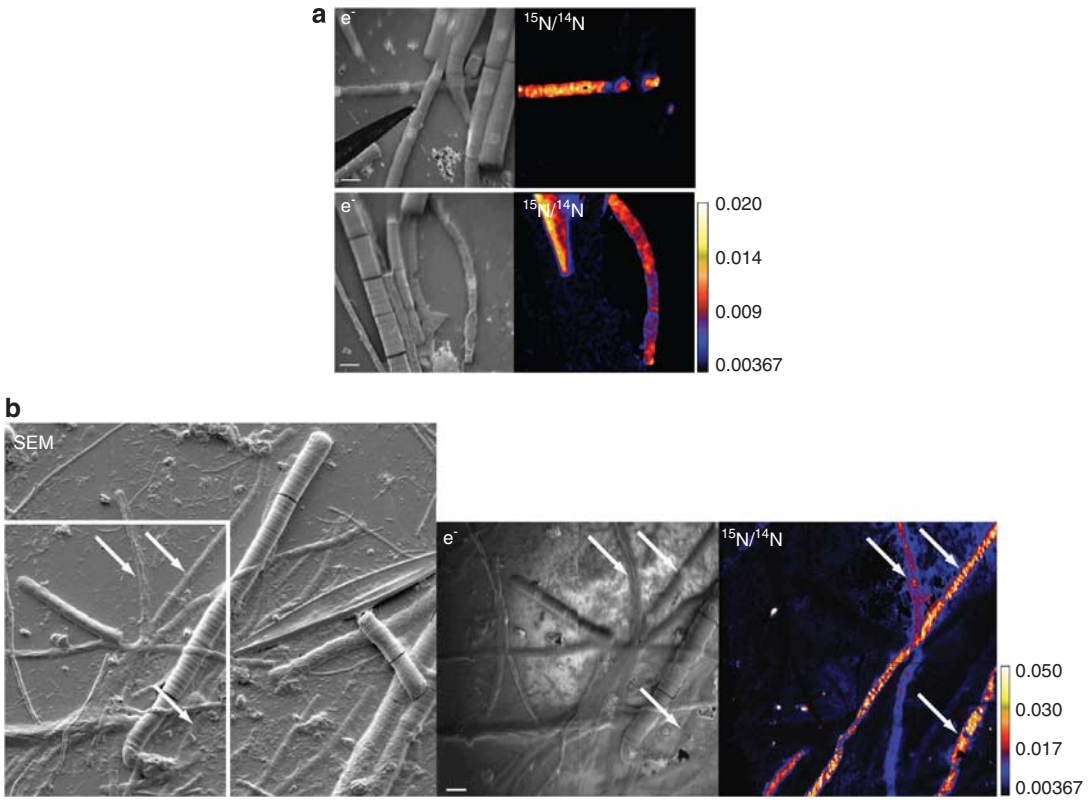

Figure 2 (**a**, **b**) Representative NanoSIMS secondary electron (e^-) and nitrogen isotope ratio ($^{15}N/^{14}N$) images of Elkhorn Slough microbial mats from October 2009 after incubation with $^{15}N_2$ in the dark. Small filamentous cyanobacteria that fixed nitrogen incorporated the ^{15}N tracer (clarified by the arrows in **b**). The color scale bar represents $^{15}N/^{14}N$ ratios (0.00367 corresponds to natural abundance). Scale bars represent 5 μm.

observed in smaller cyanobacterial filaments and ca. 20% in the single cells.

Identification of an uncharacterized nifH *phylotype in Elkhorn Slough mats*

To determine the diazotroph community in the mats and determine which community members were actively expressing the functional gene for N_2 fixation, *nifH* clone libraries were constructed from DNA and cDNA recovered from mat samples in October 2009 (21 October 2009, 2250 hours and 24 October 2009, 0310 hours). DNA-derived *nifH* sequences recovered from both time points clustered with *nifH* cluster I and cluster III of the *nifH* tree as defined previously (Chien and Zinder, 1996; Zehr *et al.*, 2003) (Table 1). Sequences of *nifH* cluster III were found to be numerically dominant in both samples: 21 October (79.6%) and 24 October (70.7%). In contrast, the majority of the expressed *nifH* sequences recovered from cDNA of these samples were found to group with cluster I (75% and 85.2%, respectively) and a minor fraction with cluster III (25% and 14.8%, respectively). Diversity indices indicated a reduced diversity of expressed *nifH* sequences (Simpson index OTU_{95}, 1.32 and 2.01, 21 and 24 October, respectively) versus sequences from *nifH* genes (Simpson index, OTU_{95} 3.17 and 2.50).

Of the expressed *nifH* sequences in samples from the 21 and 24 October, 52% and 23.9%, respectively, grouped with cyanobacterial sequences in cluster I. Almost all (97%) of these cluster I cyanobacterial sequences formed a monophyletic lineage distinct from other cyanobacterial *nifH* sequences in the database (Figure 3a). The sequence identity within this monophyletic lineage was $>94.7\%$ on the deduced amino-acid level. This cluster does not include a cultured cyanobacterium. The only closely related sequence in publicly available databases that clustered with this lineage was a translated *nifH* sequence (DQ821979) recovered from a mixed *Lyngbya* culture enriched from mats collected in Guerrero Negro, Mexico (Moisander *et al.*, 2007) (Figure 3b). The amino-acid sequences of the monophyletic lineage from Elkhorn Slough were 96.2–100% identical to this sequence.

Cultivation of a cyanobacterium belonging to the novel lineage from Elkhorn Slough

The above described NanoSIMS analysis identified small filamentous cyanobacteria to be highly active in incorporating $^{15}N_2$ into biomass. Sequencing of

Table 1 Taxonomic affiliation of *nifH* sequences from DNA and cDNA in October 2009 Elkhorn Slough microbial mat samples

	21 October 2009		*24 October 2009*	
	DNA (%)	*cDNA (%)*	*DNA (%)*	*cDNA (%)*
Cluster I	20.4	75.0	29.3	85.2
Cyanobacteria (ESFC-1-related)	3.2 (0%)	52.2 (96%)	4.0 (100%)	23.9 (100%)
γ-proteobacteria	15.0	8.7	16.0	15.9
α-proteobacteria	2.2	—	—	—
ε-proteobacteria	—	14.1	9.3	45.5
Cluster III	79.6	25.0	70.7	14.8
Unaffiliated sequences	32.3	12.0	26.7	5.7
δ-proteobacteria	47.3	13.0	32.0	9.1
Distantly related to Clostridia	—	—	12.0	—

Abbreviation: ESFC-1, Elkhorn Slough Filamentous Cyanobacterium-1.
21 October 2009, 2250-hour sample: 93 sequences (DNA) and 92 sequence (cDNA), and 24 October 2009, 0310-hour sample: 75 sequences (DNA) and 88 sequences (cDNA) were analyzed. The percentage of ESFC-1-related sequences within the cyanobacteria of the clone libraries are depicted in parantheses.

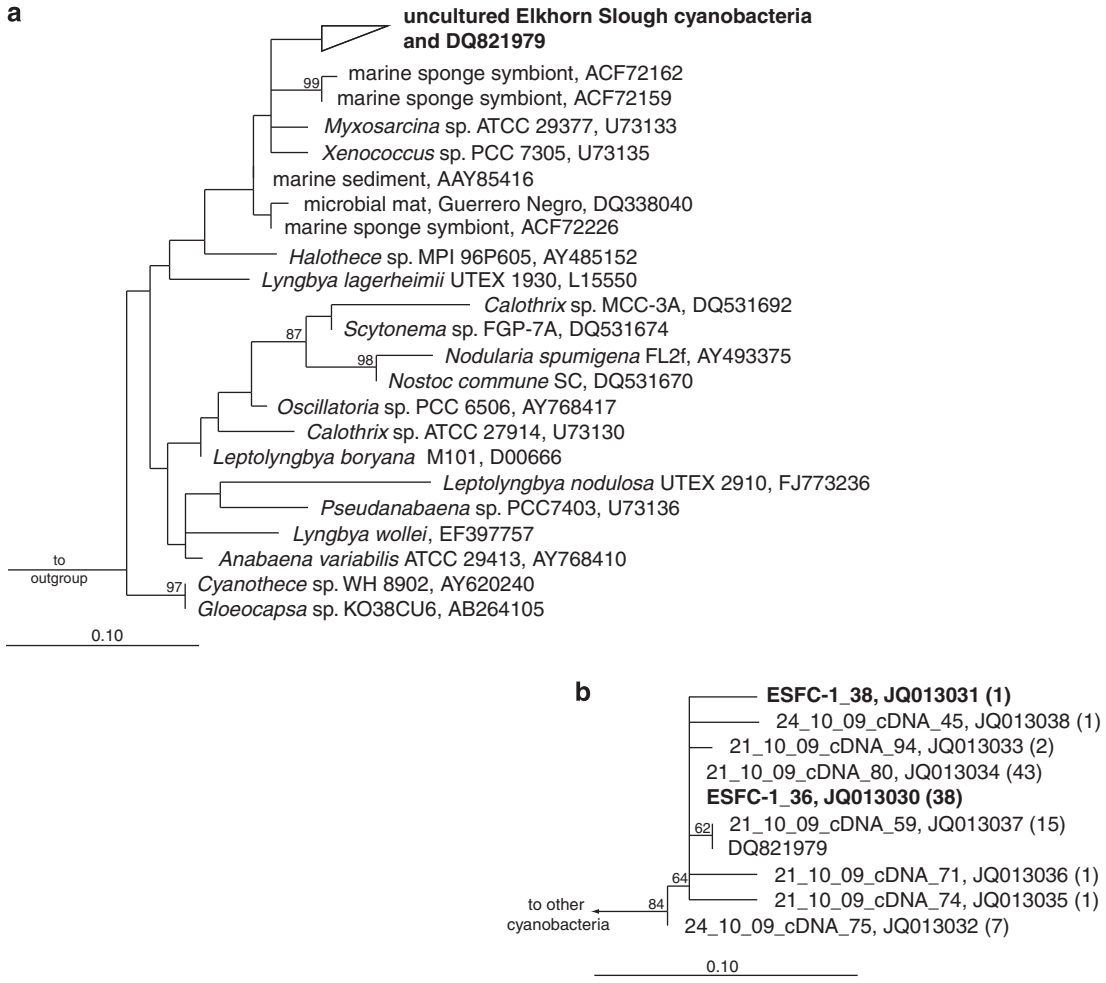

Figure 3 (**a**) Maximum likelihood tree of cyanobacterial deduced dinitrogenase reductase (*nifH*) sequences from Elkhorn Slough microbial mats collected in October 2009 (bolded) and their closest relatives. The novel lineage represents 37% of the sequences recovered from cDNA clone libraries; DQ821979 is the only closely related sequence. Bootstrap values calculated with the PhyML algorithm that were ⩾50% are displayed in the tree. Scale bar represents 10% estimated sequence divergence. (**b**) Maximum likelihood tree of the ESFC-1 *nifH* phylotypes (bolded) grouping with the cyanobacterial *nifH* sequences from Elkhorn Slough microbial mats and DQ821979. Representatives of OTU_{97} are depicted along with the number of sequences within each OTU in parentheses. Bootstrap values calculated with the PhyML algorithm that were ⩾50% are displayed in the tree. Scale bar represents 10% estimated sequence divergence.

nifH transcripts revealed that a substantial proportion of the expressed *nifH* sequences clustered in a novel cyanobacterial lineage. Therefore, we initiated cultivation experiments targeting small, non-heterocystous filamentous cyanobacteria in an attempt to identify the cyanobacteria associated with these expressed *nifH* sequences. One of these cultivations yielded a unicyanobacterial enrichment dominated by a filamentous cyanobacterium, named ESFC-1, which showed a similar morphology to the highly ^{15}N-enriched filaments visualized by NanoSIMS (Figures 2 and 4b). The maximal filament length observed in culture was 600 μm, and average cell sizes were 2.75 ± 0.07 μm length and 1.78 ± 0.02 μm width. When tested for $^{15}N_2$ incorporation by IRMS, we found that the ESFC-1

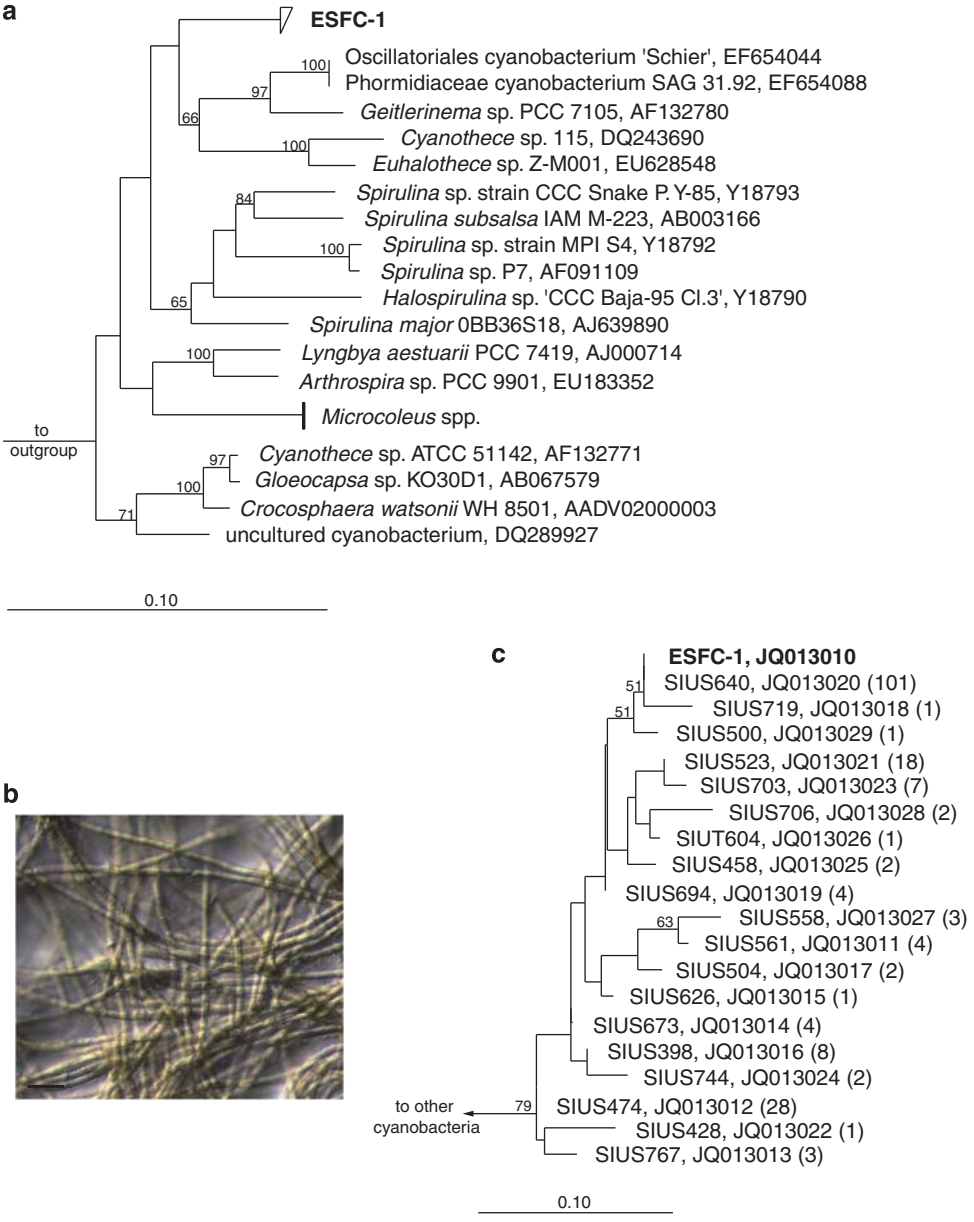

Figure 4 (**a**) Maximum likelihood tree of the cyanobacterial 16S rRNA from the ESFC-1 enrichment culture (bolded) with its closest relatives, linking the new *nifH* sequence cluster from Elkhorn Slough mats to a nearly full-length 16S rRNA gene sequence. Bootstrap values calculated with the PhyML algorithm that were ⩾50% are displayed in the tree. Scale bar represents 10% estimated sequence divergence. (**b**) Inset micrograph depicts ESFC-1 filaments from the unicyanobacterial culture. Scale bar represents 10 μm. (**c**) Maximum likelihood tree of the nearly full-length 16S rRNA gene sequence from ESFC-1 (bolded) together with the related sequences from Elkhorn Slough mats collected in January 2009, illustrating the diversity of this novel group. In all, 46% of the sequences from 12 January 2009 ('SIUS', cDNA sample, 2100 hours, 151 out of 329 sequences) and 14.8% of the sequences from 13 January 2009 ('SIUT', cDNA sample, 07:00 hours, 36 out of 243 sequences) grouped monophyletically with the 16S rRNA gene sequence type from ESFC-1. OTU_{98} representatives of Elkhorn Slough sequences along with the number of sequences within an OTU in parentheses are depicted in the tree. Bootstrap values calculated with the PhyML algorithm that were ⩾50% are displayed in the tree. Scale bar represents 10% estimated sequence divergence.

culture was highly enriched in ^{15}N after 24-h incubation ($^{15}N/^{14}N = 0.00522 \pm 4.9 \times 10^{-06}$ or $421.2 \pm 1.4‰$).

The *nifH* sequences of the ESFC-1 enrichment culture were recovered by PCR amplification and two highly similar *nifH* phylotypes were identified (OTU cut-off of 97% at the amino-acid level, 39 sequences analyzed). These sequences differed in only three amino acids and both are possibly different genomic copies of ESFC-1. Both phylotypes clustered with the novel cyanobacterial lineage from Elkhorn Slough microbial mats (Figure 3b) with up to 100% sequence identity at the deduced amino-acid level and up to 99.4% identity at the DNA level. Cyanobacterial 16S rRNA gene sequences recovered from the ESFC-1 culture yielded one phylotype (36 sequences analyzed, $\geqslant$99.6% sequence identity) (Figure 4a). The ESFC-1 16S rRNA sequence was only distantly related to sequences in publicly available databases with the closest sequence (DQ289927, 92.5% sequence identity) recovered from South Atlantic Bight sediments off the coast of Savannah, GA (Hunter *et al.*, 2006). The sequence of the closest cultured representative to ESFC-1 was *Spirulina* strain CCC Snake P. Y-85 (Y18793) (92.3% sequence identity), isolated from Yellowstone hot springs.

ESFC-1 is a representative of a prevalent and diverse cyanobacterial group

To investigate the prevalence and diversity of the novel cyanobacterial group in Elkhorn Slough mats, pyrotag sequences of V8 amplicons of the 16S rRNA gene from multiple time points in 2009 and nearly full-length sequences of the 16S rRNA gene of one particular time point were obtained. ESFC-1 affiliated sequences were identified in six out of the seven time points from samples collected throughout 2009. The sequences were proportionally higher in amplicon libraries generated from cDNA (0.28%–36%) than from DNA samples (0–5%) (Figure 5) and substantially higher in cDNA libraries recovered from mat samples collected in January 2009. Therefore, cDNA from these samples were selected to generate nearly full-length 16S rRNA clone libraries to investigate the diversity of environmental 16S rRNA sequences that clustered with the ESFC-1 cyanobacterial group. Of the 572 near full-length 16S rRNA sequences recovered from the January 2009 samples, 187 (32.7%) grouped monophyletically with the 16S rRNA gene sequence type from ESFC-1. This monophyletic clustering was observed with multiple treeing algorithms (maximum likelihood, maximum parsimony and neighbor joining) and was supported with a bootstrap value of 79% (Figure 4b). The sequence diversity within this cluster of ESFC-1 related sequences (92.5–100%) revealed a great diversity of ESFC-1-related cyanobacteria.

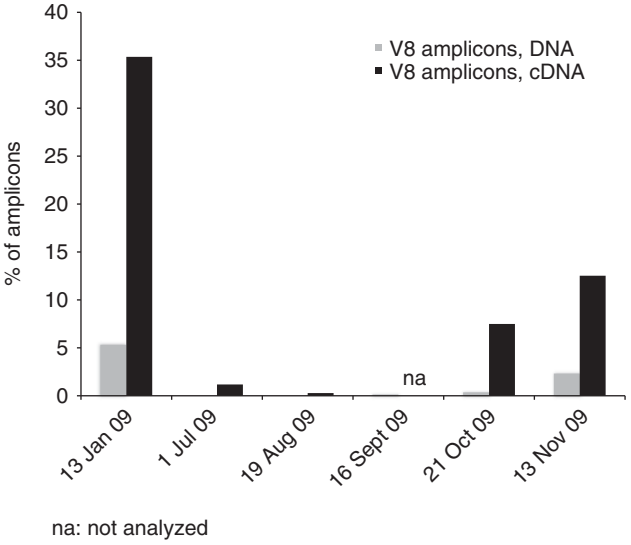

Figure 5 Relative abundance of ESFC-1 affiliated 16S rRNA V8 pyrotag amplicons in 2009. Sequences affiliated with ESFC-1 were found in samples from 13 January (0700 hours), 1st July (0400 hours), 19 August (0115 hours), 16 September (1200 hours), 21 October (2250 hours) and 13 November (0000 hours) with a higher proportion in cDNA samples compared with DNA samples.

Investigation of in situ *N_2 fixation by ESFC-1 and related cyanobacteria*

To confirm that cyanobacteria of the ESFC-1 cluster were actively incorporating $^{15}N_2$ as suggested by *nifH* sequencing and our initial NanoSIMS survey of the October 2009 samples, further NanoSIMS investigations were combined with CARD-FISH. Oligonucleotide probes specific for the 16S rRNA of the ESFC-1 cluster identified in the January 2009 samples were designed and stringent hybridization conditions for the newly designed probes were determined as described in Supplementary Information (Supplementary Table 1 and Supplementary Figures 5 and 6). Samples from October 2009 incubated with $^{15}N_2$ were hybridized with the ESFC-1-specific probes and imaged by epifluorescence microscopy. CARD-FISH combined with NanoSIMS analysis demonstrated that filaments of the ESFC-1 cluster were enriched in ^{15}N (Figure 6). In all, 63% of 76 analyzed ESFC-1 ROIs were significantly enriched in ^{15}N based on a 95% confidence interval, with levels ranging from low enrichments ($^{15}N/^{14}N = 0.00374$ or 19.3‰) to a $^{15}N/^{14}N$ ratio of 0.025 (5802.3‰).

Discussion

In this study, we used a combination of biogeochemical, molecular and NanoSIMS analysis in conjunction with targeted cultivation experiments to investigate the active diazotrophic community in microbial mats from the Elkhorn Slough estuary. With this approach, we discovered a novel filamentous cyanobacterial group, ESFC-1, that represented

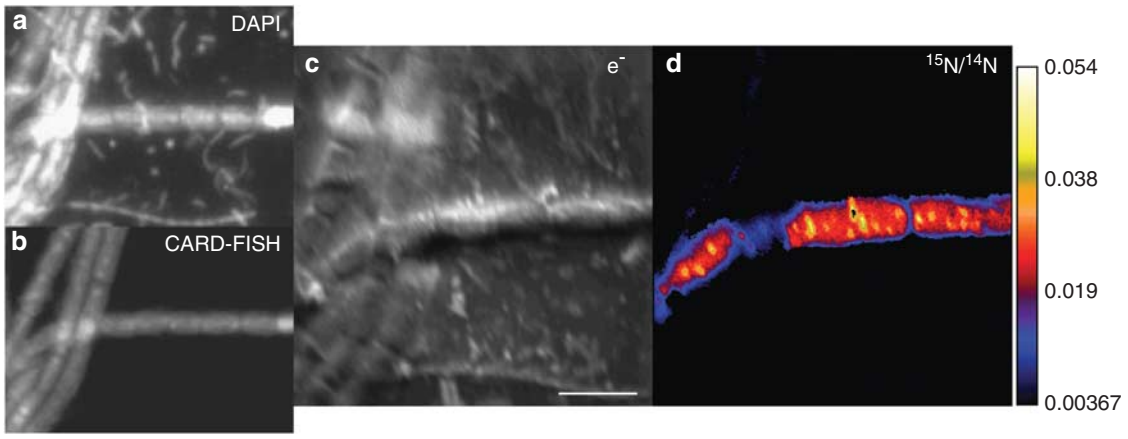

Figure 6 NanoSIMS and epifluorescence images of filaments stained with the ESFC-1-specific CARD-FISH probe ESFC-1_177 after incubation of mat cores with $^{15}N_2$. (**a**) Epifluorescence image of DAPI-stained cells and filaments. (**b**) Epifluorescence image of filaments stained with ESFC-1-specific probe ESFC1_177. (**c**) Secondary electron (e^-) image of the same region. (**d**) Nitrogen isotope ratio ($^{15}N/^{14}N$) image depicting an ESFC1_177-stained filament highly enriched in ^{15}N ($^{15}N/^{14}N$ 0.025). Scale bar represents 5 μm.

almost the entire fraction of the cyanobacterial expressed *nifH* genes. This expression data along with NanoSIMS analysis of ESFC-1 and other mat cyanobacteria led us to conclude that members of this novel group were the most active N_2-fixing cyanobacteria in the investigated mat.

Biogeochemical analysis of the mats collected in October 2009 revealed patterns of N_2 fixation activity similar to those observed previously in mats from Guerrero Negro in Mexico, Bird Shoal in North Carolina, and the island Mellum in the North Sea, which were dominated by filamentous non-heterocystous cyanobacteria; nitrogenase activity was restricted to the upper 2 mm of the mat (Stal *et al.*, 1984) and the highest at night under anoxic conditions (Stal *et al.*, 1984; Bebout *et al.*, 1987, 1993; Paerl *et al.*, 1996; Omoregie *et al.*, 2004a, b). Despite these biogeochemical parallels, molecular data indicated that a previously unrecognized group of diazotrophs was present and highly active in Elkhorn Slough mats.

Overall, the *nifH* gene sequences that we obtained from the Elkhorn Slough mats grouped with the *nifH* clusters I and III. This pattern was previously observed in mats from Guerrero Negro and the island Schiermonnikoog in the Wadden Sea (Omoregie *et al.*, 2004a, b; Moisander *et al.*, 2006; Severin *et al.*, 2010). A reduction in *nifH* transcript diversity compared with *nifH* gene diversity was observed in clone libraries recovered from Elkhorn Slough. The same pattern was previously reported in mats from Guerrero Negro (Moisander *et al.*, 2006). These observations support the hypothesis that only a subset of the microbial community with the genetic potential for N_2 fixation actively expresses *nifH* in these cyanobacterial mats. Interestingly, a high number of the transcripts from Elkhorn Slough in October 2009 formed a novel monophyletic sequence cluster, suggesting that a new cyanobacterial group might be an important diazotroph in these mats. However, N_2 fixation is regulated on multiple levels ranging from transcription (Chen *et al.*, 1998) to posttranslational protein modification (Kim *et al.*, 1999), so the detection of *nifH* gene transcripts does not necessarily imply active N_2 fixation *in situ*.

NanoSIMS investigations provided additional data to support the assignment of a highly active diazotrophic cyanobacterial group in Elkhorn Slough mats predicted by marker gene analysis. NanoSIMS of $^{15}N_2$-incubated Elkhorn Slough mat samples from October 2009 showed that among the filamentous cyanobacteria, a particular morphotype that had cells of ca. 2 μm width and ca. 3 μm length was highly enriched in ^{15}N. We hypothesized that these cyanobacterial filaments might harbor the novel and highly expressed *nifH* sequences. To identify these cyanobacteria, we conducted targeted cultivation experiments for diazotrophic cyanobacteria searching for the suspected morphotype, and indeed, we obtained a similar morphotype (ESFC-1) in one enrichment that fixed $^{15}N_2$ in culture. Cyanobacteria in this culture harbored the novel *nifH* sequence type and enabled us to link the *nifH* sequence to the corresponding 16S rRNA gene sequence. These data also enabled us to design specific oligonucleotide probes for CARD-FISH/NanoSIMS experiments to examine ^{15}N incorporation into the ESFC-1 populations in $^{15}N_2$-incubated Elkhorn Slough mats. These studies revealed that ca. 60% of the ESFC-1-related filaments actively incorporated ^{15}N. However, ^{15}N enrichment in these populations was highly variable, with a small fraction of the ESFC-1 community dominating the total ^{15}N incorporation. Large variations in ^{15}N enrichments have previously been noted in NanoSIMS investigation of *Chlorobium phaeobacteroides*-related cells in Lake Cadagno (Switzerland) (Halm *et al.*, 2009), *Aphanizomenon* sp. in the Baltic Sea (Ploug *et al.*, 2010) and shipworm symbionts (Lechene *et al.*, 2007). This variability may be due to spatial heterogeneity in the local environment, such as small-scale variations in nutrient concentrations,

diffusion rates or redox potentials. The microbial mat environment is highly heterogeneous (Jørgensen *et al.*, 1983; Des Marais, 2003), and as filaments experience different environmental conditions, they may differ in their physiology and N_2 fixation activity, resulting in different ^{15}N enrichment levels. We noticed that even adjacent filaments (Figure 6) differed in the degree of ^{15}N-enrichment, suggesting that the local environment may not be solely responsible for the variable activity of the filaments, but also could be due to strain or species-level variation in ESFC-1 populations. The high level of diversity in the ESFC-1 clade at the 16S rRNA level supports this hypothesis. The functional significance of such fine scale variation has been extensively studied in low-diversity acid mine drainage biofilms and wastewater treatment reactors (Denef *et al.*, 2010). Furthermore, the filaments could be in different physiological states with only a part of the population actively growing. These data also illustrate the power of single-cell techniques to reveal functional heterogeneity at the single-cell level within closely related populations.

Amplicon pyrosequencing of the V8 region across multiple samples throughout the year 2009 revealed that the ESFC-1 cluster is prevalent in Elkhorn Slough mats. ESFC-1-related sequences were much less abundant in the total microbial community (DNA) compared with the active community (cDNA), representing an example of a low abundance member of a microbial community that may perform an important ecosystem function, as was observed in NanoSIMS investigations of phototrophic bacteria in a meromictic lake (Musat *et al.*, 2008; Halm *et al.*, 2009). An ESFC-1-related *nifH* gene sequence has also been detected in a mixed *Lyngbya* culture enriched from mats collected in Guerrero Negro, Mexico (Moisander *et al.*, 2007) suggesting that ESFC-1-related cyanobacteria may be present in other microbial mats, but may not have been identified due to low abundance at the time of sampling. The pyrotag sequence data were supported by near full-length 16S rRNA sequences from the January 2009 cDNA samples, in which ca. 33% of all cDNA sequences (187 total sequences) formed a broad monophyletic clade with a degree of diversity that may represent multiple species. Deep sequencing of mat samples from other environments may identify ESFC-1-related populations and help broaden our understanding of the ecophysiology of this novel cyanobacterial group.

Interestingly, we found no evidence that *Microcoleus* spp., which are ubiquitous and abundant members of marine microbial mats globally, had an important role in N_2 fixation in the Elkhorn Slough mats. *Microcoleus chthonoplastes*, the type strain for mat-dwelling *Microcoleus* spp., was for many years not thought to be a diazotrophic cyanobacterium. However, the recently completed genome of *M. chthonoplastes* PCC 7420 revealed a *nif*-gene cluster (Bolhuis *et al.*, 2010), which grouped the *nifH* gene with δ-proteobacterial genes in cluster III. In the *nifH* clone library of DNA samples from one of the two October time points (24 October 2009), we identified three sequences that grouped closely with these *M. chthonoplastes* sequences in cluster III. However, no *nifH* transcripts related to *Microcoleus* spp. were recovered from the Elkhorn Slough mat samples described in this study. Additionally, very low levels of ^{15}N enrichment (average $^{15}N/^{14}N$ ratio of $0.00382 \pm 2.4 \times 10^{-5}$ or 40.9 ± 6.6‰) in *Microcoleus* spp. filaments were observed in NanoSIMS analysis of $^{15}N_2$-incubated mat samples from October 2009 with the highest $^{15}N/^{14}N$ ratio being 0.00401. This trend was also noted in other samples with nighttime N_2 fixation (December 2007) in which *Microcoleus* spp. filaments had enrichment values very close to natural abundance ($^{15}N/^{14}N$ $0.00368 \pm 2.4 \times 10^{-5}$, 3.4 ± 6.6‰). These low levels of ^{15}N enrichment observed may be due to cross-feeding from active diazotrophs. In contrast, ESFC-1 filaments were observed with $^{15}N/^{14}N$ ratios up to 0.025 (5802‰). Although *Microcoleus* spp. may fix N_2 under specialized conditions, we could not find evidence that *Microcoleus* spp. were actively fixing $^{15}N_2$ in the investigated Elkhorn Slough mats. This finding lends further support to our hypothesis that ESFC-1-related cyanobacteria were the dominant active cyanobacterial diazotrophs in the investigated Elkhorn Slough microbial mats.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)